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Differential expression of salivary (Amy_1) and pancreatic (Amy_2) human amylase loci in prenatal and postnatal development

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Summary. The age-dependent development of α-amylase expression *in utero* and during the first two years of life is reported. Separation of salivary and pancreatic amylase isozymes in a discontinuous buffered sheet polyacrylamide electrophoretic system, with subsequent densitometry, provides a reliable semiquantitative method of estimating the proportions of salivary and pancreatic amylases in urine and amniotic fluid samples. In the newborn the predominant amylase isozymes seen in the urine are of salivary origin. As the child ages the level of amylase in the urine rises and an increase in the proportion of pancreatic amylase isozymes occurs. Amniotic fluids of late first and early second trimester pregnancies contain salivary isozymes. None of the amniotic fluid samples examined has pancreatic amylase isozymes. These data reflect a differential development of the expression of the two amylase loci as the child ages. The pancreatic (Amy_2) proportion of the total urinary amylase approaches adult levels by 16 months of age. Conversely, the salivary (Amy_1) locus is expressed as early as 18 weeks of gestation and remains relatively constant with but a small increase in salivary amylase (units/ml) activity during early development, as the total amylase activity approaches adult values.

Human α-amylases (α-1,4-glucan 4-glucanohydrolase; E.C. 3.2.1.1) are the products of two tightly linked loci, Amy_1 primarily expressed in the salivary glands and Amy_2 in the pancreas (Merritt, Rivas, and Ward, 1972; Karn *et al*, 1975). Both loci lead to the production of complex families of isozymes that exhibit distinctly different electrophoretic mobilities. The sheet polyacrylamide electrophoretic systems developed in our laboratory allow resolution, differentiation, and direct comparison of all isozymes in serum and urine and assignment by type (Merritt *et al*, 1973b; Karn *et al*, 1975). Methods for quantifying salivary and pancreatic amylase in human biological specimens have been explored and a reliable densitometric semiquantitative method developed.

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Previous studies of amniotic fluid (Laxová, 1972) and sera (Kamarýt and Fintajslová, 1970) used agar electrophoresis on 2.54 cm slides, which precluded direct comparison of multiple samples, and considered the allelic expression from each locus to be a single isozyme. Their conclusions are misleading (Merritt *et al*, 1973b). A third study (Wolf and Taussig, 1973) investigated amylase expression in amniotic fluid using disc electrophoresis. Resolution of the isozymes was improved but direct comparison of isozyme migration distances was difficult and resulted in misinterpretation of the pancreatic or salivary origin of the isozyme.

We report here the age-dependent expression of Amy_1 and Amy_2 in amniotic fluids and in urines of children from birth to 24 months of age and compare it with pancreatic and salivary amylase expression in adults.

Methods and materials

Random urine samples were collected from normal healthy infants (N = 202), ranging from newborn to 24 months. Urine was used because collection is easy and amylase is usually more concentrated in urine than in serum. Samples were either frozen and stored at −20° C for subsequent evaluation (storage periods never exceeded 4 months) or a few crystals of thymol were added before storage at 4° C. Amniotic fluids from late first, early second, and late third trimester pregnancies were collected, and stored frozen for periods ranging up to 4 months (N = 26). Neither freezing nor the addition of thymol had any apparent effect on subsequent electrophoresis or amylase quantitation.

Amylase assays

Amylase activity was measured by a modification of the saccharogenic serum amylase method of Searcy, Haysahi, and Berk (1966). Amyloclastic methods were less satisfactory in detecting amylase at the low levels of the enzyme seen in infant urine. Urine (0.25 ml) was added: (1) to an assay tube containing a 1.0 ml starch solution; and (2) to a zero time tube with a 1.0 ml starch solution and 2.0 ml dinitrosalicylic acid reagent (DNSA). Both were incubated 60 minutes at 37° C. The reaction was stopped by addition of 2.0 ml of the DNSA solution. Colour was developed in assay and control tubes by placing them in a boiling water bath for precisely 5 minutes. Reaction mixtures were diluted 1/10 with distilled water and read at 540 nm using a Beckman DBG spectrophotometer and compared with a standard curve. A unit of enzyme activity was defined as 1.0 mg of maltose produced during the 60 minutes sample incubation. All quantitative values are expressed as units per ml.

Electrophoresis

The details of the multiple sample, vertical sheet polyacrylamide electrophoretic system for saliva and urine have been reported (Ward, Merritt, and Bixler, 1971; Merritt *et al*, 1973b). Samples were applied 8 to 10 cm from the cathodal end of a 12 × 27 cm gel, the sample slots sealed with petrolatum, and the gel covered with Saran wrap. Vertical electrophoresis was performed at a constant current of 20 mA per gel and terminated when the buffer front reached a distance 15 to 17 cm from the origin, in approximately 17 hours. The slowest isozyme bands were found 5 to 7 cm and the fastest isozymes 10 to 14 cm from the sample origin. The incubation times in the starch substrate solution varied inversely with the amylase activity of the sample. Infant urine and amniotic fluid required 45 minutes' incubation in soluble starch solution followed by a brief water rinse and incubation for up to 2 hours at 37° C before staining. The isozyme patterns were developed with I_2-KI solution and photographed. Anodic disc electrophoresis was also employed according to the method of Davis (1964). The running gel was incubated directly in the 1 per cent buffered starch solution for 45 minutes to 1 hour, depending on whether the sample was urine or amniotic fluid, to develop zymograms as reported by Wolf and Taussig (1973).

Densitometric techniques

Gels were photographed using transmitted light with a Polaroid MP-3 camera loaded with type 55 P/N film. The image of each sheet gel zymogram channel (10 mm width) on the Polaroid negative was analysed with an RFT gel scanner (Transidyne General Corp., Ann Arbor, Mich.) using transmitted light at 510 nm and a 0.5 × 8.0 mm slit. Since the gel background is differentially stained from origin to buffer front, the uneven baseline precludes use of the RFT gel scanner integrator. Therefore, the baseline was drawn, and the area under each isozyme peak determined by counting the graph paper squares under the separate peaks, allowing improved, proportionally quantitative results. Unpublished experimental data on Polaroid negative exposure time, gel inclubation, and amylase isozyme sample dilution all showed a consistent and statistically significant ratio for the densitometric areas of the Pa A1 isozyme to the Pa A2 isozyme.

Results

Infant urine

Urines from 202 individuals were analysed. Though approximately 20 Amy_2 variant phenotypes were expected, as calculated from the frequency of Amy_2 variants in adults (0.10—Merritt *et al*, 1973b) none was detected ($P = (0.9)^{202} = 5.71 \times 10^{-10}$). This result was not unexpected as it is shown below that 29% of the infants and children had no detectable zymogram and 38% had not developed pancreatic sufficiency to the extent required to detect Amy_2 isozymes. Among the 67 infants remaining no variants were detected though 7 would have been expected ($P = (0.9)^{67} = 8.60 \times 10^{-4}$). This is in keeping with the observation that the variant isozymes are generally present in decreased amounts in adult heterozygotes. Only those children over 12 months of age have near adult values of total amylase activity (see Fig. 6); therefore, little or no variant isozyme manifestations were expected.

Amylase zymograms on acrylamide gels with their corresponding densitometric scans, including salivary (Sa) amylase, pancreatic (Pa) amylase, infant and adult urine, and amniotic fluid from 2nd and 3rd trimester pregnancies are shown in Fig. 1. The amylase type of each of the isozymes in the urine samples can be assessed from the zymograms (Merritt *et al*, 1973b). As previously reported (Karn *et al*, 1975) the order of the isozymes in the common phenotypes Amy_1 A (salivary) and Amy_2 A (pancreatic), numbered from the origin towards the anode, is Pa A1, Sa A1, Pa A2, Sa A2, Sa A3, Pa A3, and Sa A4.

Several isozymes have very similar electrophoretic mobilities (Fig. 2). The major components of

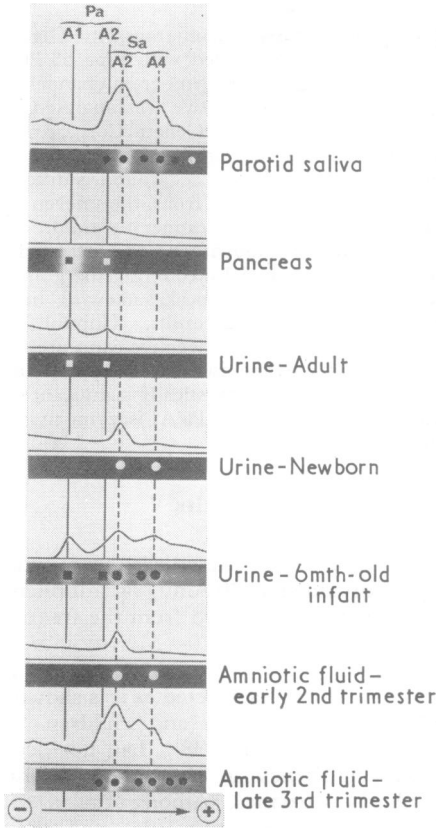

FIG. 1. Sheet polyacrylamide gel zymograms of human α-amylase from various tissues and secretions (see methods and materials). The relative migration of the two cathodal isozymes of pancreatic amylase, Pa A1 and Pa A2 (solid lines), and the two primary salivary isozymes, Sa A2 and Sa A4 (dotted lines), are shown for the various tissues. If the isozyme appears in any of the samples it is indicated as a square for the pancreatic type isozymes (□, ■), and a circle for the salivary type isozymes (○, ●).

salivary and pancreatic amylase zymogram activities are the first 4 salivary isozymes and the first 2 pancreatic isozymes, respectively. The more distal bands (Pa A3, Sa A5, Sa A6) seen in adult urine or saliva are rarely seen in infant and early childhood (to 15 months) urine or in amniotic fluid. Though the isozymes are frequently visible on direct observation (Fig. 1), densitometric scanning was not wholly satisfactory. Therefore, the ratio of Pa A1 to Pa A2 was calculated (see below) to determine the relative amount of Pa A2 present in the Sa A1, Pa A2, Sa A2 region.

Among the thousands of normal fresh adult urines and hundreds of pancreatic extracts that have been analysed in this laboratory, the proximal isozyme Pa A1 is the most intense, with the more

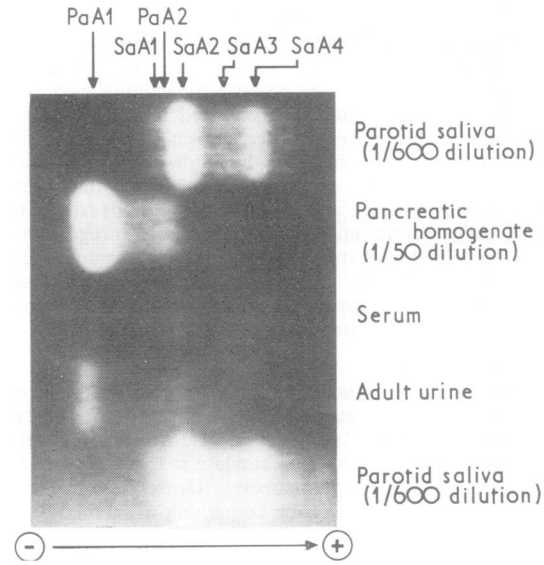

FIG. 2. Sheet polyacrylamide gel zymogram showing the primary tissue sources of human amylase. The pancreatic (Pa) and salivary (Sa) type isozymes are labelled indicating their relative rates of migration.

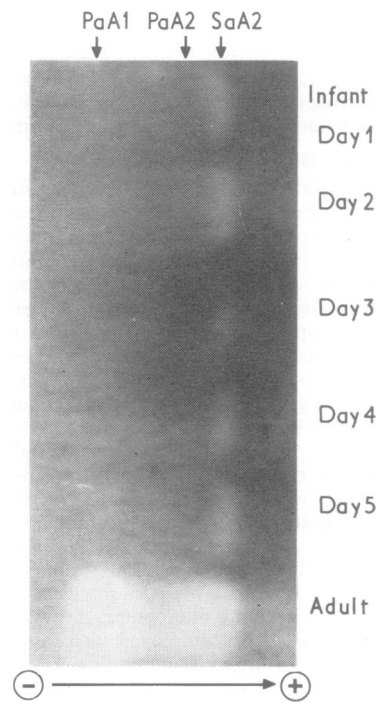

FIG. 3. Polyacrylamide sheet gel zymogram showing a single infant's unconcentrated urinary amylase pattern over a 5-day period as compared with that of a normal adult urinary amylase pattern. No pancreatic isozymes are seen in the infant's urine in this short time.

distal Pa A2 and Pa A3 bands decreasing in intensity. Previous work in this and other laboratories (Keller *et al*, 1971) has shown that this manifestation of a heavy primary cathodal isozyme with its subsequent lighter anodal bands is probably the result of a partial deamidation of the enzyme (Karn *et al*, 1975). Fresh pancreatic homogenates have a larger Pa A1 to Pa A2 activity ratio than that found in adult urine, presumably because of differential deamidation.

That amylase expression in the urine of individual children remained relatively constant over a period of several days was shown by electrophoretic analysis of samples collected daily from a 5-month-old infant during a 5-day period (Fig. 3). The amylase pattern observed was of the salivary type, i.e. Pa A1 is absent and the major component migrated in the Sa A2 region. The total amylase concentration and, therefore, the zymogram pattern, varies slightly in intensity each day, probably depending on the fluid intake and renal function of the child. However, the various amylase isozymes do not suddenly appear and/or vanish.

Urines from children of various ages were arbitrarily grouped: newborn to 5 days, 5 days to 1 month, 2 to 3, 4 to 5, 6 to 7, 8 to 9, 10 to 11, and 12 to 15 months, over 16 months, and adults. The amylase pattern of each sample was scored either as no zymogram activity or, if there was an electrophoretic pattern, according to the presence or absence of Pa A1. When Pa A1 was not present, all isozymes were salivary in type.

Total amylase activity was assayed in each sample and the average for the age group calculated (Table). This average included samples with no zymogram activity. Approximately 50% of samples showed no visible amylase electrophoretic pattern in the newborn to 5-day-old age-group. By the age of 12 months, however, all the children tested showed zymogram activity (Fig. 4). The newborn to 5-day-old group shows the Pa A1 isozyme in 25% of the urines. The proportion increases to 100% in those over 1 year of age (Fig. 4). The total amylase activity calculated for the 1 to 2 month age-group is 0.53 ± 0.38 units; values increase to 2.44 ± 0.492 units in those over 16 months. (Normal adult urine amylase assayed by this system varied widely from 0.45 units to over 3.50 units of activity, mean = 2.17 units $\pm$ 1.14 units.) In Fig. 5, amylase activity is plotted as a function of age and a regression analysis was performed on the data points (correlation coefficient = 0.51, $P < 0.001$).

A variant of human amylase was studied to estimate the proportion of total pancreatic amylase activity represented by the Pa A2 isozyme in urine, without the interference of Sa A2 band activity. An individual with salivary amylase phenotype

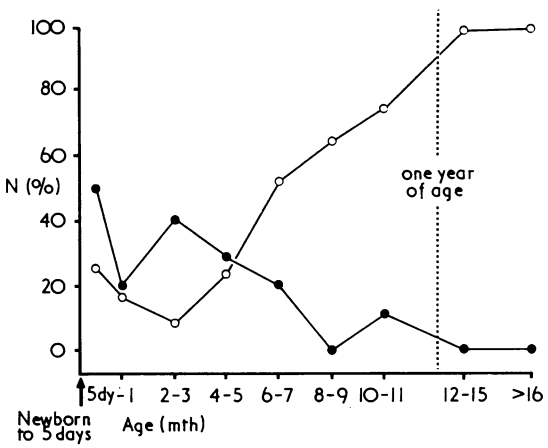

FIG. 4. A plot of the percentage in each age-group with no detectable zymogram (●) and the percentage in each age-group with pancreatic type amylase zymogram activity (○).

TABLE

Age-group	No. of Individuals (N)	% of Group with no Zymogram Activity	% of Group Showing Pancreatic Amylase	Average Total Activity (assayed) (mg maltose 60 min)*
Newborn–5 d	36	50	25	0.58 ±0.41
5 d–1 mth	28	21	17	0.532±0.38
2 mth–3 mth	41	41	9	0.71 ±0.65
4 mth–5 mth	37	29	24	0.81 ±0.661
6 mth–7 mth	28	21	53	0.70 ±0.415
8 mth–9 mth	14	0	64	1.41 ±0.604
10 mth–11 mth	8	12	75	1.62 ±0.397
12 mth–15 mth	5	0	100	2.22 ±0.481
> 16 mth of age	5	0	100	2.44 ±0.492
Adult	16	0	100	2.17 ±1.14

* Includes all of the individuals in each age-group.
± Standard deviation.

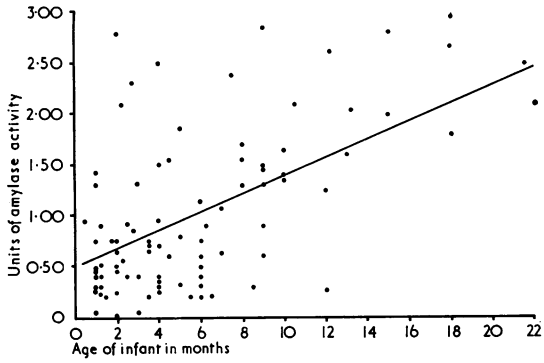

FIG. 5. The total assayed amylase activity of urine samples plotted as a function of age. The solid line is a linear regression least squares fit to the points shown, with a coefficient of coincidence of 0.51 ($P<0.001$).

Amy_1 r and pancreatic amylase phenotype Amy_2 A was used. The Amy_1 r phenotype has a pattern similar to that of the Amy_1 A phenotype but with an anodal shift. Since the predominant isozyme species of Amy_1 r migrates near the normal isozyme Sa A4 of Amy_1 A (Karn *et al*, 1973), the pancreatic isozyme band Pa A2 cannot be mistaken for the salivary isozymes in such individuals. A consistent quantitative relation between the amount of Pa A1 and Pa A2 in the adult urine was found on densitometric examination. The ratio of Pa A2 to Pa A1 is 31.5%. For any given infant urine that shows a Pa A1 band, an estimate of the size of the Pa A2 band which lies within the next anodal band can be made. This estimate assumes that the 31.5% Pa A1 to Pa A2 ratio is valid in infants as well as in adults. The total amylase activity and the calculated pancreatic activity are shown for the various age groups in Fig. 6. The difference between the curves represents salivary amylase. It does not appear that the activity attributable to salivary type isozymes increases with age. In general this appears to be the case; however, we should remember that the proportion with no amylase activity on the zymogram falls from 50% to 0% by the age of 12 months. This represents a minor but disproportionately lower increase in salivary type isozymes when compared with the rate of increase in pancreatic type isozymes. None the less by the age of 12 months the amount of salivary type amylase activity is 0.978 units, which is similar to that seen in adult urine, 0.868 units.

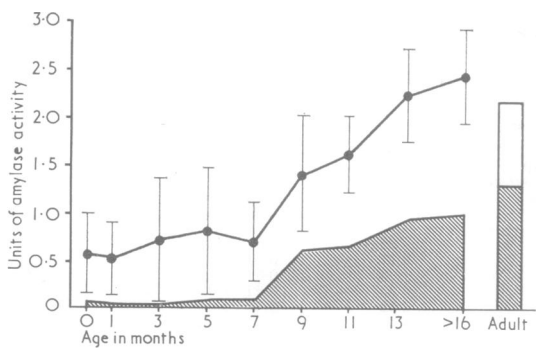

FIG. 6. The total assayed amylase activity averaged for various age-groups of infants and children shown with the standard deviation for each group (●). The calculated average amount of pancreatic amylase for each age-group (see results section) is shown as the cross-hatched area.

Amniotic fluid

Eight out of ten amniotic fluid samples from first trimester pregnancies produced faint salivary amylase zymogram patterns; the other two showed no zymogram activity (mean 1.06 ± 0.64 amylase units). Zymogram intensity increases as does the assayed activity by the third trimester (mean 1.94 ± 0.54 amylase units). Pancreatic type isozymes have not been observed in any of the 26 amniotic fluid samples.

Comparative disc and sheet gel electrophoresis of a 3rd trimester amniotic fluid with relatively high amylase activity, mixed with adult urine is shown in Fig. 7. The zymograms from disc electrophoresis (Fig. 7, B-6) showed two predominant isozymes similar to urine or serum, and it was only after electrophoresis side by side in sheet acrylamide (Fig. 7, A-6) that it was clear that the primary

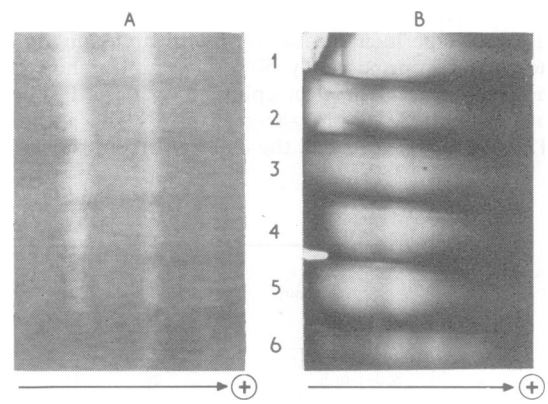

FIG. 7. A sheet polyacrylamide gel zymogram (A) and anodic disc gel zymograms (B) of mixtures of amniotic fluid and adult urine. Gels are shown with the same samples applied to both electrophoretic systems. Sample 1, adult urine; sample 2, adult urine: 3rd trimester amniotic fluid, 4:1; sample 3, 3:2; sample 4, 2:3; sample 5, 1:4; sample 6, 3rd trimester amniotic fluid. Adult urine shows the Pa A1 band and the Pa A2, Sa A2 composite band in both gel systems. The amniotic fluid shows the Sa A2 and Sa A4 isozymes only. No pancreatic amylase isozymes are seen in amniotic fluid.

cathodal isozyme was Sa A2 and not Pa A1. When amniotic fluids from 1st trimester pregnancies were compared electrophoretically in the same gel with a normal control serum and the mother's serum obtained at the time of amniocentesis, the maternal serum not only contained more amylase activity than the amniotic fluid, but also Pa A1.

Discussion

Previous investigators employed an agar gel electrophoresis system to describe the differential development of salivary and pancreatic amylase in infant serum during the first year of life (Kamarýt and Fintajslová, 1970). As has been shown (Merritt *et al*, 1973b) the cathodal isozyme of a nonvariant individual is entirely of pancreatic origin while the anodal two or three bands are in fact mixtures of isozymes of pancreatic and salivary type. In a comparison of the agar and polyacrylamide gel systems (Merritt *et al*, 1973b), it was shown that the cathodal salivary isozyme in the agar system corresponds to Sa A1 and Sa A2 in the acrylamide gel system, and the more anodal bands of salivary isozymes seen in the agar system are Sa A3–A4 and Sa A5–A6. The two isozymes in the pancreatic series seen in agar patterns are Pa A1 and Pa A2, the latter migrating slightly less anodally than Sa A2. In the agar gel electrophoretic system in which samples are often analysed two at a time, as in the studies of Kamarýt and Laxová (1965), Pa A2 and Sa A2 are not distinguished. When Kamarýt and Fintajslová (1970) reviewed their data from electrophoretic patterns of infant serum, they assumed that all isozymes anodal to Pa A1 were of salivary origin. Therefore, the isozyme Pa A2 was incorrectly assigned to the salivary type amylase isozyme family.

Sheet acrylamide electrophoresis indicated that salivary and pancreatic isozymes did not have the same electrophoretic mobilities (Merritt *et al*, 1973b). On the other hand, mixtures of salivary and pancreatic amylase isozymes in a sample are not always resolved even in the polyacrylamide gel systems. As previously discussed (Results section) we have been unable to resolve unequivocally Pa A2 from the slow salivary type isozymes, Sa A1 and Aa A2, and thus have elected to evaluate only the presence or absence of Pa A1 in each sample. The presence or absence of pancreatic type amylase has been expressed as the percentage of the total number of samples in chosen age-groups. In Fig. 2 the percentage of samples in each succeeding age-group that lacks zymogram activity decreases. That is to say the expression of pancreatic amylase increases with age.

By inferring the activity of the Pa A2 band, described in the Results section, a percentage of the total assayed amylase activity contributed by the pancreatic isozymes was determined and, as seen in Fig. 6, total amylase and pancreatic amylase activity in the urine rises with age. The pancreatic amylase isozyme activity varies from 0.03 unit in the newborn to 5-day-old group to 1.45 units in the over 16-month group, an increase of approximately fifty-fold. The rise in calculated salivary type isozymes in the urine from 0.55 unit in the newborn to 5-day group to 1.27 in the over 16-month group, a 23% increase, is less striking.

The data reported here, as well as those of others (Kamarýt and Fintajslová, 1970), suggest an age-dependent differential development of the expression of the Amy_1 and Amy_2 genes in infants. However, our results suggest that their calculation of the relative amylase isozyme components from agar electrophoresis is in error because of their assumption that all of the anodal bands in agar electrophoresis were of salivary type rather than mixtures. This assumption resulted in an overestimate of salivary type and underestimate of pancreatic type amylase isozyme components when both were present. Kamarýt and Fintajslová (1970) estimated that in children of the oldest age-group in their study (age 1 year), two-thirds of the total urinary amylase isozymes were of salivary type, and one-third of pancreatic type. This is not a critical error in infants of less than 12 months of age when salivary isozymes contribute 60% and pancreatic 40% of the total amylase activity by our methods. It becomes very misleading after that time as pancreatic type isozymes continue to increase, then stabilize at approximately 60% of the total activity in the adult. The second anodal band on agar electrophoresis increases in intensity with the ageing of the child, because of the presence of an increased amount of pancreatic isozyme (Pa A2) in the second band and not, as Kamarýt and Fintajslová (1970) claim, from increased salivary isozyme expression. Therefore, an estimate of the salivary amylase component in adult urine by the method of Kamarýt would show a much lower percentage of pancreatic isozymes and a higher estimate of the salivary isozymes than is actually present.

Laxová (1972) employed the same agar electrophoretic system as Kamarýt and Fintajslová in classifying amniotic fluid amylase isozymes as to type. It is reported that 6% of the samples examined exhibited a salivary variant pattern based on an isozyme which migrates more anodally than the principle Amy_1 isozymes. The anodal isozyme is not a variant isozyme but a component of the

Amy_1 A phenotype as previously shown (Merritt *et al*, 1973b). All heterozygous Amy_1 variants described to date are phenotypically expressed as cathodal isozyme families (Merritt *et al*, 1973b; Karn *et al*, 1975). Further, it has been shown that each amylase allele produces a single peptide which is modified post-translationally into distinct isozyme families. Therefore, the isozyme Laxová (1972) refers to as variant (Amy_1^B) is not an independently segregating allele but the anodal salivary isozymes Sa A3–A4. Notwithstanding these minor criticisms we agree with Laxová's (1972) interpretation that amniotic fluid does not contain pancreatic amylase isozymes in sufficient quantity to be detected. Our studies indicate that salivary amylase (Amy_1) expression is well developed at birth, the onset of expression apparently occurring as early as the first trimester of gestation. Other authors as well as our own laboratory results indicate that pancreatic amylase (Amy_2) has not been observed in amniotic fluid and is virtually absent from sera and a significant number of urines at birth (Laxová, 1972).

This is in contradiction to the reports of Wolf and Taussig (1973), who reported pancreatic amylase isozymes in all 1st and 3rd trimester amniotic fluids they examined. Since Wolf and Taussig employed disc gel zymograms the difficulties in comparing individual amylase isozymes make their conclusion suspect (see Results section).

These data probably reflect two associated phenomena: (1) the infant kidney gains competency during the first year of life, its ability to concentrate urine improves—as reflected by an increase in urine osmolality with age (Rahill, 1969); (2) the developing infant pancreas is beginning to express the pancreatic amylase gene—a reflection of the onset of pancreatic competency. This is consistent with absorption studies of various lipid, protein, and carbohydrate diets, which have shown that the levels of the pancreatic digestive enzymes are still developing during the first few months of life (De Vizia *et al*, 1975). Other authors have found a similar increase in total amylase activity per gram of cadaver pancreas obtained from newborn, infant, and other young children (Matas *et al*, 1975).

Of the two identified amylase loci in humans, the pancreas, of endodermal derivation, expresses the Amy_2 locus exclusively while the salivary glands, of ectodermal origin, express the Amy_1 locus. These data seem to indicate that ectodermally derived tissue sources of amylase production, such as the salivary glands, are active much sooner in fetal development than those corresponding endodermally derived tissue sources of amylase production such as the pancreas.

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